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The effect of oral phenytoin and vitamin C therapy on enterocutaneous fistula healing

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ABSTRACT

Introduction: Phenytoin is reported to heal the enterocutaneous fistula. Meanwhile, vitamin C is known to improve wound healing. The comparison of phenytoin and vitamin C in the treatment of enterocutaneous fistula is never reported.
Methods: A randomized controlled trial with a post-test-only group design using artificial enterocutaneous fistula *Ratus Norvegicus* male Wistar rats were reported. Rats were divided into four groups randomly, namely, Control (K), Treatment 1: combined phenytoin and vitamin C (P1), Treatment 2: phenytoin (P2), and Treatment 3: vitamin C (P3). Variables under study were a number of fibroblast and angiogenesis. One Way Anova test, followed by the Post-Hoc Test, was used to test the difference among and between groups.
Results: The mean value of fibroblasts in groups K (501.86 ± 107.10), P1 (861.53 ± 25.99), P2 (719.93 ± 24.61), P3 (781.46 ± 28.23). The mean value of angiogenesis in groups K (18.63 ± 4.24), P1 (48.86 ± 10.23), P2 (26.26 ± 1.64), P3 (31.96 ± 2.97). The Post-Hoc test showed that were significant differences in the number of fibroblasts and angiogenesis from the group given a combination of oral phenytoin and vitamin C therapy compared to the group given oral phenytoin, vitamin C, and control ($p < 0.05$).
Conclusion: A combination of oral phenytoin and vitamin C therapy increased the number of fibroblasts and angiogenesis of enterocutaneous fistula in Wistar rats, better than single.

Keywords: *angiogenesis, enterocutaneous fistula, fibroblasts, phenytoin, vitamin C.*
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INTRODUCTION

Enterocutaneous fistula (ECF) is described as a tragedy or disaster in the surgical field.[1] ECF is an abnormal condition with a connection between the intra-abdominal gastrointestinal tract and the skin.[2] ECF is classified as low output (<200 ml / day), moderate output (200-500 ml / day), and high output (> 500 ml / day).[3] The incidence of traumatic ECF has increased due to the higher incidence of surgery, especially for trauma cases.[1] The ECF mortality rate varies from 6% to 33%. Incidence depends on the etiology. Infected pancreatic necrosis has a very high 50% incidence. Trauma patients have an incidence of 2% to 25%, and abdominal sepsis has an incidence of 20% to 25%. It is estimated that 80% of enterocutaneous fistulas are of secondary iatrogenic origin after surgery. Surgical complications, such as enterotomy or bowel anastomosis dehiscence, are known to be at high risk for the development of enterocutaneous fistulas. Meanwhile, 20% of non-operative fistulas are caused by systemic diseases such as Crohn's disease, radiation enteritis, malignancy, trauma, or ischemia.[4]

ECF is a rare and poorly studied post-traumatic complication associated with significant morbidity. One study of 2,373 patients requiring laparotomy surgery performed by Teixeira and colleagues found that post-traumatic ECF development was associated with a significant increase in length of intensive care (ICU) (28.5 ± 30.5 vs 7.6 ± 9.3 days, $p = 0.004$), length of stay in hospital (82.1 ± 100.8 vs 16.2 ± 17.3 days, $p < 0.001$) and mean hospital costs ($ 539,309 vs $ 126,996, $p < 0.001$).[5]

The development of multidisciplinary ECF therapy remains a problem and a challenge with a 5-15% mortality rate.[2] Comprehensive wound care accompanied by Total Parenteral Nutrition (TPN) is currently the choice of therapy, with fistula closure without surgery increasing from 19% to 92%.[6] In addition, ECF that does not heal is associated with foreign components. Radiation, inflammation, infection, inflammatory bowel disease, epithelization of the fistula tract, neoplasms, distal obstructions, and steroids (FRIENDS) are indications for surgical intervention. However, the operation must be performed in a timely manner, after the patient's general condition is good and there has been lysis of the intra-abdominal fibrous adhesions from the previous operation.[1]

Wound care at ECF at this time also has many treatment methods that can be done with moist dressings using negative pressure wound therapies (NPWT) or one of which is often known as vacuum-assisted closure (VAC). Current use of VAC is reported to improve the quality of life for EFC patients, where the skin around the ECF wound is protected.[7] Wainstein et al., in their case series, demonstrated the effectiveness of VAC in 98% of the patients, with a significant reduction in fistula output after the first day to 7 days of treatment.[7,8] VAC reduces pro-inflammatory cytokines and the expression of matrix metalloproteinases

(MMPs), including MMP-1, 2, 13, which play a role in degrading collagen.[9-11] In addition, there is an increase in the expression of vascular endothelial growth hormone (VEGF) in this therapy.[10] The cost of implementing this method is high and should be done with the expertise of trained stoma nurses.

Phenytoin, which has long been known as an anti-seizure drug, is currently reported from several studies showing it has a therapeutic effect on wound healing.[12] Several studies have shown that phenytoin can improve wound healing and speed up fibrosis. Phenytoin's mechanism for accelerating wound healing is still not fully understood, but several theories have been proposed. The mechanisms by which phenytoin induces wound healing include stimulating fibroblast proliferation, increasing angiogenesis, increasing granulation tissue formation, decreasing collagenase activity, increasing collagen deposits, inhibiting glucocorticoid activity, decreasing exudate in wounds and inhibiting antibacterial activity either directly or indirectly by affecting cells, inflammation and neovascularization. There is also evidence that phenytoin may play a role in the healing of pilonidal sinus fistula and wounds and that such a positive effect on wound healing can be applied to the healing of gastrointestinal fistulas.[13-15] In another study, it was found that phenytoin could reduce levels of MMP-1, MMP3, MMP-9, and TNF-α but did not decrease IL-6, whereas IL-6 could increase fibroblast proliferation and collagen synthesis.[16] This mechanism becomes the basis for its application in ECF cases, with an increase in the number of fibroblasts, angiogenesis, and collagen synthesis, which will allow the closure of the fistula tract.

Vitamin C, also known as ascorbic acid (AA), is involved in all phases of wound healing. In the inflammatory phase, it is necessary for apoptosis and neutrophil clearance. During the proliferation phase, AA contributes to the synthesis, maturation, secretion and degradation of collagen.[17] Vitamin C is a cofactor for collagen synthesis, and a major antioxidant consumed rapidly after injury. Vitamin C supplementation is important for the timely stopping of the inflammatory phase, increasing fibroblast migration, matrix deposition and neovascularization in wound healing through modulation of transcript levels of HO-1, TGF-β, CTGF and VEGF. Vitamin C facilitates wound healing by a pleiotropic mechanism that goes beyond its role in collagen metabolism. In several studies, vitamin C has been shown to suppress the pro-inflammatory process by pleiotropic mechanisms while promoting anti-inflammatory and pro-resolution effects in macrophages. Vitamin C is also closely involved in collagen metabolism and regulation. Therefore much research has focused on the role of vitamin C in wound healing.[18]

In a study on vitamin C in epilepsy, it was found that the absorption of vitamin C was not impaired during treatment with phenytoin, whereas the mean value of vitamin C was not lower in patients who had received phenytoin therapy for a period of 2 years compared to untreated ones.[19] In addition, vitamin C has also been shown to have a hepatoprotective effect, which can provide significant protection against the side effects of phenytoin, which are toxic to the liver, by its ability to improve lipid peroxidation through free radical scavenging activity.[20]

From the explanation above, research on the effects combination of phenytoin and vitamin C on wound healing in ECF needs to be further studied by giving it orally, which has never been done at this time. This study will compare the use of a combination of phenytoin-vitamin C and phenytoin or vitamin C alone. The marker used to see wound healing is the presence of an increase in the number of fibroblasts and angiogenesis.

METHODS

This study was an experimental animal study with a randomized controlled trial with a post-test-only group design. The research sample was male Wistar strain *Ratus norvegicus* rats and was divided randomly into four groups, namely, the control group (K), treatment 1 (P1), treatment 2 (P2), and treatment 3 (P3). Each group consisted of 6 rats according to the WHO sample size. The samples used met the inclusion criteria: male rats aged 8-10 weeks underwent the procedure for making enterocutaneous fistulas, had no anatomical abnormalities and had an average body weight was 150-200 grams after acclimatization. The mice that looked sick (inactive movement) experienced a weight loss of> 10%, and the mice that died at the time of the study were excluded.

Data collection was carried out for 3 months, from April to June 2020. Treatment of mice and the process of preparing and taking the tissue was carried out at the Biosciences Institute, Universitas Brawijaya, Malang. The process of making paraffin blocks and HE staining was carried out at the Faculty of Medicine, Anatomical Pathology Department, Universitas Brawijaya, Malang. Pathologic slides were read in the Anatomical Pathology laboratory of Universitas Brawijaya, Malang and the Anatomical Pathology laboratory of the Universitas Sultan Agung (UNISSULA) Faculty of Medicine, Semarang.

A total of 24 male Wistar rats aged 8-10 weeks with a body weight of 150-200 grams were acclimatized in the laboratory for one week, then enterocutaneous fistulas were created. The fistula was made by enterotomy with a size of 5 mm in the caecum through the access of the lower left side of the abdominal mouse with a size of 7 mm. The enterotomy was sutured with PGA 5.0 to the abdominal wall to form an enterocutaneous fistula. After this procedure, the rats will be randomly divided into 4 groups, with 6 rats in each group. Every day, rats' fistula wounds were treated with moist gauze (K), a combination of oral phenytoin and oral vitamin C with moist gauze (P1), oral phenytoin with moist gauze (P2), and oral vitamin C with moist gauze (P3). Oral phenytoin at a dose of 0.6 mg / grBW is dissolved with aquabidest and given once a day through an oral sonde. Oral vitamin C with a dose of 0.09 mg / grBW was dissolved with aquabidest and given once a day through an oral sonde. All subjects will be treated equally both in terms of maintenance and nutrition. On the 7th day, the termination will be carried out on all mice. Termination was performed by cervical dislocation under anesthesia. The sample was then sliced for slide preparation and examined for microscopic preparations with HE staining.

The variables in this study consisted of independent variables, namely oral phenytoin and or oral vitamin C. The dependent variables in this study were the number of fibroblasts and angiogenesis on tissue histopathology preparations. The assessment of the number of fibroblasts and angiogenesis was carried out by 2 anatomical pathologists independently. The results of the assessment of 2 anatomical pathologists were carried out by the intra-class correlation coefficient (ICC) test to assess the agreement in providing an assessment. The ICC results were obtained on the assessment of the number of fibroblasts 0.983 and angiogenesis 0.977 (confidence interval> 95%) without knowing the treatment of the study subjects.

Before data analysis, data were cleaned, coding and tabulation. As all data were normally distributed, the results were assessed with mean ± standard deviation. The test used in this study was the One Way Anova test, followed by a Post-Hoc Test to assess differences between groups. The results were significant if $p \leq 0.05$ with the 95% confidence interval not exceeding. Data analysis was performed using SPSS.

Ethics was obtained from the Ethical Commission for Health Research, Faculty of Medicine, Universitas Diponegoro, with the Ethical Clearance number No. 68/EC/H/FK.UNDIP/VII/2020. All experimental animals were cared for and managed accordingly to animal maintenance standards.

RESULTS

Twenty-four male Wistar rats were treated with fistula wound treatment with moist gauze (K), combined phenytoin and vitamin C with moist gauze (P1), oral phenytoin with moist gauze (P2), and oral vitamin C with moist gauze (P3). All Wistar rats were still alive and healthy at the end of the study. Therefore statistical analysis was done on all Wistar rats.

The rats came from the same line, namely the Wistar line, male gender and the rat body weight data were normally distributed and homogeneous (Table 1).

Data for the variable number of fibroblasts obtained a mean number of 501.86 ± 107.10 in the group without giving any therapy (K), 861.53 ± 25.99 in the group administered with an oral combination of phenytoin and vitamin C (P1), 719.93 ± 24.61 in the group administered with oral phenytoin therapy (P2), and 781.46 ± 28.23 in the group administered with oral vitamin C therapy (P3) (Figure 1).

The lowest number of fibroblasts was seen in the control group (without therapy) by 501.86 ± 107.10, while the highest number of fibroblasts was seen in the combination of oral phenytoin and oral vitamin C, by 861.53 ± 25.99. Based on the One way ANOVA test, it can be seen that the p-value <0.05, which means that there is a significant difference. In the Post-Hoc test, there were differences in the number of fibroblasts from the group given oral combination therapy of phenytoin and oral vitamin C, oral phenytoin only, and oral vitamin C only, which had significant differences compared to the control group in each group (p <0.05), and there was a significant difference in the group given the combination of oral phenytoin and oral vitamin C compared to the group given oral phenytoin or oral vitamin C alone (p <0.05) (Figure 2).

Data on the number of angiogenesis variables obtained a mean number of 18.63 ± 4.24 in the group without giving therapy (K), 48.86 ± 10.23 in the group giving oral phenytoin therapy and oral vitamin C (P1), 26.26 ± 1.64 in the group giving oral phenytoin therapy (P2), and 31.96 ± 2.97 in the group given oral vitamin C therapy (P3) (Figure 3).

The lowest amount of angiogenesis in the control group was 18.63 ± 4.24, while the highest amount of angiogenesis was seen in the treatment group with a combination of oral phenytoin and oral vitamin C, 48.86 ± 10.23 (Figure 4). Based on the One way ANOVA test, the p-value was <0.05, which means that the differences are significant. In the Post-Hoc test, the amount of angiogenesis of the group given oral combination therapy of phenytoin and oral vitamin C, oral phenytoin, and oral vitamin C had a significant difference compared to the control group (p <0.05), and There was a significant difference in the group given the combination of oral phenytoin and oral vitamin C compared to the group given single oral phenytoin or vitamin C only (p <0.05).

DISCUSSION

In this study, fistula tissue was taken on the 7th day after the animal was given the treatment in each group, which this time is the end of the inflammatory phase of the wound healing process. The number of fibroblasts has increased by the end of this time.[21] In the treatment group, there was a significant difference in the number of fibroblasts, with the group administered with the combination of oral phenytoin and oral vitamin C having the highest number. Meanwhile, the group that was not given oral phenytoin therapy and oral vitamin C had the lowest number of fibroblasts. From these results, it was found that there was a significant difference in the number of fibroblasts between the groups that were not given therapy and the groups that were given therapy.

The increase in the number of fibroblasts in the administration of oral

Table 1. Descriptive table of normality and homogeneity of body weight.

Groups	Body weight (Gram)		*p normality (Shapiro-Wilk)*	Homogeneity (Levene Statistic)
	Mean ± SD	Median (min-max)		
K	180.87 ± 11.54	181.95 (167.58 – 196.83)	0.632	0.235**
P1	184.39 ± 7.55	185.24 (172.48 – 192.29)	0.604	
P2	175.65 ± 7.11	173.22 (168.82 – 185.65)	0.202	
P3	178.88 ± 6.52	179.07 (167.47 – 187.53)	0.559	

Note: *Significant (p < 0.05); **Homogen (p > 0.05)

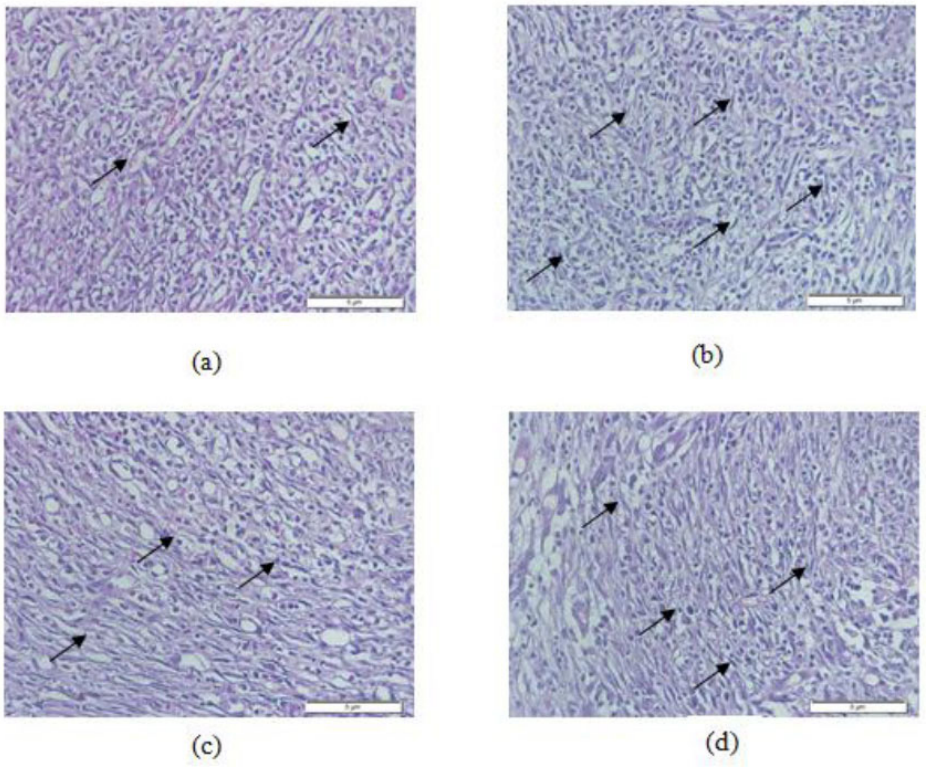

Figure 1. Histopathological picture of the number of fibroblasts (black arrow) with hematoxylin-eosin staining at 400x magnification. **A)** Without treatment; **B)** Combination of oral phenytoin and vitamin C; **C)** Oral phenytoin, and; **D)** Oral vitamin C.

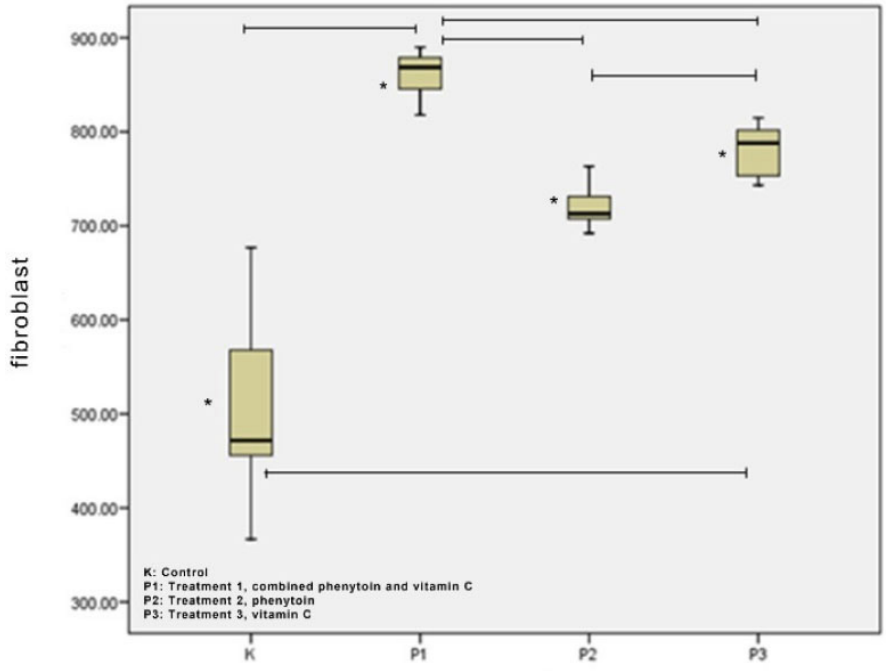

Figure 2. Boxplot graph of the number of fibroblasts from each group. Control (K), Treatment 1: combined phenytoin and vitamin C (P1), Treatment 2: phenytoin (P2), Treatment 3: vitamin C (P3). There was a statistically significant difference (One Way ANOVA P <0.05). And also obtained significant differences when compared in each group (Post-Hoc test); * **significant P <0.05**.

phenytoin therapy and oral vitamin C showed the effect of phenytoin and vitamin C therapy on enterocutaneous fistulas. This was in accordance with the theory that the administration of phenytoin and vitamin C had a stimulating effect on fibroblast proliferation.[14-16,18] Increased proliferation of fibroblasts in enterocutaneous fistula wounds induced by phenytoin and vitamin C would have a positive effect on the spontaneous closure of enterocutaneous fistulas. One of the factors that were expected to occur in fistula closure therapy was the presence of fibroblasts to support the wound healing process.[22] In the current popular conventional therapy for enterocutaneous fistulas using VAC, which in its application has a mechanism to reduce pro-inflammatory cytokines, reduce the expression of matrix metalloproteinases (MMPs), including MMP-1, 2, 13, which play a role in degrading collagen, so that wound healing is inhibited. as well as inducing micro deformation on the wound surface and complex wound healing.[9-11,23] Lu Feng et al. showed that micro deformation in wound healing was associated with fibroblast induction.[23] The use of VAC could improve the quality of life for patients with enterocutaneous fistulas, where there was the skin around the fistula wound is protected.[7] Wainstein et al., in their case series study, demonstrated the effectiveness of VAC used in 98% of the patients, with a significant reduction in fistula output after the first to the seventh day of treatment.[7,8] In addition, there was an increase in the expression of vascular endothelial growth hormone (VEGF) in this therapy.[10] The positive effect of differences in the number of fibroblasts in this study could be a factor in the spontaneous closure of enterocutaneous fistulas. The study from Jaber was reported on a case study conducted with intravenous phenytoin giving a positive effect on the healing of gastrointestinal fistulas. In this study, Jaber used systemic therapy because the healing process was occurring primarily in fibrosis in the fistula tract, which could be achieved through systemic therapy than topical therapy. Applied to the wound surface of the fistula.[24] In several studies, vitamin C has been shown to suppress the pro-inflammatory process by

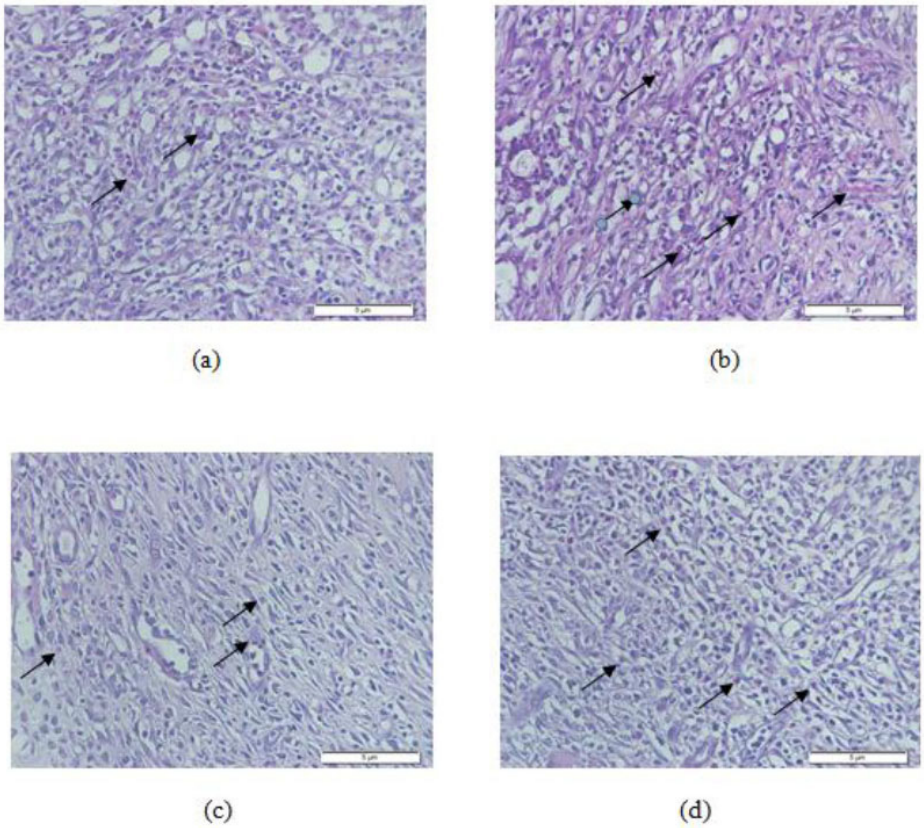

Figure 3. Histopathological picture of the amount of angiogenesis (black arrow) with hematoxylin-eosin staining at 400x magnification. **A)** Without treatment; **B)** Combination of oral phenytoin and oral vitamin C; **C)** Oral phenytoin, and; **D)** Oral vitamin C. Arrows indicate angiogenesis.

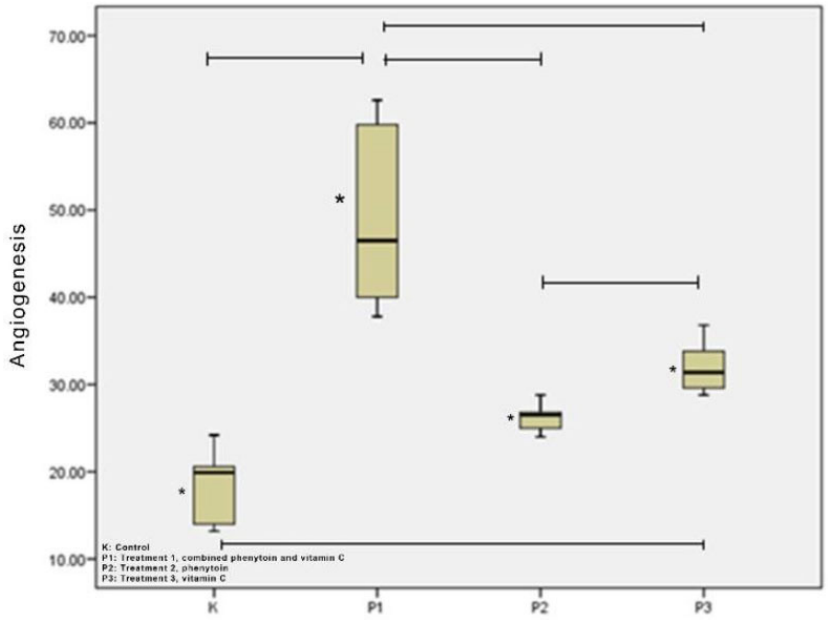

Figure 4. Boxplot graph of the amount of angiogenesis of each group. Control (K), Treatment 1: combined phenytoin and vitamin C (P1), Treatment 2: phenytoin (P2), Treatment 3: vitamin C (P3). There were statistically significant differences (One Way ANOVA P <0.05) and also obtained significant differences when compared in each group (Post Hoc test); ***significant (P <0.05)**.

pleiotropic mechanisms while promoting anti-inflammatory and pro-resolution effects in macrophages. Vitamin C is also closely involved in collagen metabolism and regulation. Therefore much research has focused on the role of vitamin C in wound healing.[18] Moreover, in the presence of phenytoin, myofibroblast and fibroblast proliferation, the production of the extracellular matrix and its protein, the activity of growth factors and their mediators may be increased within the wound. Overall, phenytoin appears to reduce collagenase activity, edema, wound exudates and bacterial load. On the other hand, ascorbic acid plays a critical role in wound repair and the healing or regeneration process as it stimulates collagen synthesis. It has been suggested that there will be rapid utilization of ascorbic acid for the synthesis of collagen at the site of a wound during the postoperative period. This explains why the combination of phenytoin and vitamin C is more powerful in the wound-healing process rather than in a single regime.

This study also assessed the amount of angiogenesis in the skin tissue on the 7th day after treatment in each group. It was found that the highest value for angiogenesis was in the group with the combination of oral phenytoin and oral vitamin C therapy. Meanwhile, the group that was not given oral phenytoin therapy and oral vitamin C had the lowest angiogenesis amount. From these results, it was found that there was a significant difference in the amount of angiogenesis between the groups that were not given therapy and the groups that were given therapy.

Angiogenesis is one of the important factors in the tissue's ability to repair itself, remove debris, and distribute nutrients and oxygen for the formation of new healthy tissue. The formation of granulation tissue, which is a dense tissue of blood vessels, macrophages, and fibroblasts embedded in a loose matrix of fibronectin, hyaluronic acid, and collagen, relies on the vascularity of the wound tissue and begins to appear in the wound about four days after injury. During the angiogenesis process, the number of capillaries which is in the extracellular matrix becomes increased due to endothelial cell proliferation.[25]

The higher amount of angiogenesis in

the treatment group given oral phenytoin and oral vitamin C therapy proved the effect of phenytoin and vitamin C therapy which induces macrophages, especially M2 stimulation, to increase various growth factor expressions. They are VEGF and PDGF, which play a role in increasing the number of angiogenesis.[26-28] In the process of spontaneous fistula closure, angiogenesis was also needed so that collagen deposits and granulation tissue could occur.[10]

Research on the benefits of giving phenytoin and vitamin C can still be extended in future studies. The limitation of this study was the researchers only observed the fistula healing process until the seventh day of treatment, where the wound healing phase was still ongoing. Observation of fibroblasts and angiogenesis by histopathological examination in this study can still be improved by using immunohistochemical methods. So that in the future, research can be carried out until the wound healing phases take place perfectly with a longer treatment time.

CONCLUSION

Supplementation of oral phenytoin and oral vitamin C alone or in combination increased the number of fibroblasts and angiogenesis of enterocutaneous fistula in Wistar rats (*Ratus norvegicus*); combination administration is better than single.

ETHICAL CONSIDERATION

Ethics was obtained from the Ethical Commission for Health Research, Faculty of Medicine, Universitas Diponegoro, with the Ethical Clearance number No. 68/EC/H/FK.UNDIP/VII/2020.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHOR'S CONTRIBUTION

YS: Planned the study, collected the data, performed the analysis, and wrote the manuscript; TN, EM, AU, IR: critically revised the draft for important intellectual content and finally approved the manuscript. All authors read and approved the final manuscript.

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